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EFFECTS OF TOTAL-BODY X-IRRADIATION ON PERITONEAL CELLS OF MICE

by
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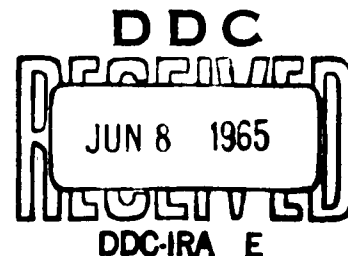
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ABSTRACT

Peritoneal cells of normal and irradiated LAF_1 mice which had not been pretreated with peritoneal irritants were studied by phase-contrast microscopy and in stained smears. 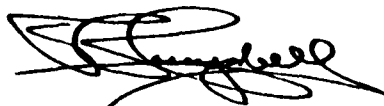The average number of leucocytes recovered per normal mouse was 7.4×10^6. Approximately 95% of the cells were mononuclear; of these, macrophages comprised 29% (2.2×10^6), small and medium lymphocytes 71% (as determined by phase-contrast). One day after whole-body exposure to 690 R ($LD_{30/30}$) or 720 R ($LD_{50/30}$), the mean leucocyte count was 3.4×10^6, macrophages accounted for 65% (2.2×10^6) of the cells. From 3 to 21 days postirradiation, cell counts gradually decreased further to 1.5×10^6. Macrophages decreased at the same rate; they constituted approximately 70% of the cells during this period. Thereafter, the number of leucocytes increased and reached normal levels by the 12th week postirradiation.

When suspensions of viable <u>Escherichia</u> <u>coli</u> or <u>Staphylococcus</u> <u>aureus</u> were injected 30 minutes before sacrifice, the macrophages of both normal and irradiated mice contained large numbers of microorganisms. 30 minutes after injection of bacteria or <u>E</u>. <u>coli</u> lipopolysaccharide (10 µg) into normal mice, the number of cells in peritoneal washings was not altered. However, after injection of these agents into irradiated mice, total cell counts were significantly lower than in uninjected or diluent-injected mice.

NON-TECHNICAL SUMMARY

The Problem

It is well established that whole-body exposure to ionizing radiation causes severe damage to the bone marrow and lymphoid tissues. As a result, the number of leucocytes in the blood is greatly diminished. It was of interest to find out whether the cells present in the peritoneal cavity, some of which originate in the lymphoid tissue, others presumably in the bone marrow, are similarly affected.

The present investigation was undertaken to determine the number and kinds of leucocytes normally present in the peritoneal cavity of the mouse and to study the effects of total body X-irradiation on this cell population.

The Findings

At least 95% of the cells in the peritoneal cavity of normal LAF_1 mice were mononuclear; of these about 70% were lymphocytes, 30% were macrophages. One day after exposure to midlethal doses of X-irradiation and for 3 weeks thereafter, both the total number of cells and the percentage of lymphocytes were greatly reduced. The number of macrophages in the peritoneal cavity declined only slightly and very gradually between 1 and 21 days postirradiation. Normal cell counts were again found approximately 3 months after exposure.

When suspensions of certain bacteria were injected into the peri-
toneal cavity shortly before collection of the cells, the macrophages
of both normal and irradiated mice contained many ingested microorganisms.
Thirty minutes after injection of bacteria or bacterial endotoxin into
normal mice, the number of cells obtained in peritoneal washings was
not altered. However, after injection of these agents into irradiated
mice, total cell counts were significatnly lower than in uninjected or
diluent-injected mice.

INTRODUCTION

It is well recognized that hematopoiesis is impaired following total-body exposure to ionizing radiations in the midlethal dose range. In support of this view may be cited observations on the histopathologic changes in the bone marrow and lymphoid tissues (1), the fall of leucocytes in the peripheral blood (2), the reduced ability to mobilize granulocytes after an appropriate stimulus (3).

One population of leucocytes which is easily accessible but which has not yet been studied systematically after irradiation, are the free cells in the peritoneal cavity. The morphology of these cells in normal mice has been described in detail by Felix and Dalton (4); quantitative data on the different cell types present may be found in reports by Goodman (5, 6) and Balner (7).

Investigators who collected peritoneal fluid cells from normal and irradiated animals for various experimental purposes reported that the yield of cells was lower after irradiation (7, 8) and that the percentage of small cells was decreased (7). Bercovici and Graham (9) described the changes in total cell population and in cell types during the 24 hours immediately following local irradiation of the abdominal area of mice.

The present investigation was undertaken to determine the number and kinds of leucocytes normally present in the peritoneal cavity of

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LAF_1 mice and to study the effects of midlethal doses of total-body X-irradiation on this cell population.

MATERIALS AND METHODS

Mice

Female LAF_1 (C57♀ x A/He♂) mice from our laboratory colony were used in all experiments. They were housed in metal cages, food pellets and acidified drinking water (pH 2.5) were supplied ad libitum.

Irradiation

A single dose of total-body X-irradiation was delivered to mice between 12 and 16 weeks of age. Irradiation factors were 250 kvp, 15 ma, filter 0.5 mm Cu and 1 mm Al, HVL 1.28 mm Cu, TSD 40 inches, dose rate 27 R per minute. The X-ray doses employed, 690 and 720 R, represented an $LD_{30/30}$ and an $LD_{50/30}$, respectively.

Harvest of peritoneal cells

Mice were killed by cervical dislocation, the skin over the peritoneum was reflected and 2 ml sterile Tyrode's solution (without Ca^{++}) containing 10 units heparin/ml were injected into the cavity. After gentle abdominal massage to dislodge the peritoneal cells, as much fluid as possible was withdrawn with a pasteur pipette and placed in a siliconized graduated centrifuge tube immersed in an ice bath. For each experiment, the washings of 4-5 mice were pooled. Bloody exudates were discarded.

Cell counts

Leucocytes were counted in a hemacytometer, viability of the cells was determined by trypan blue exclusion. The number of leucocytes harvested per mouse was calculated from the hemacytometer count and the volume of cell suspension collected.

Preparation of stained smears

The cell suspensions were centrifuged lightly in the cold (275 g for 10 min), the sediments were resuspended in 40% normal rat serum and smears were prepared with cover slips to minimize cell breakage. The slides were fixed in methanol and stained with Giemsa.

For every experiment, at least 400 cells on each of two duplicate slides were counted independently by both investigators. Individual results were in close agreement. The average of all counts was recorded.

Bacteria

A strain of Escherichia coli isolated from a normal mouse and a strain of Staphylococcus aureus from a human abscess were used. Overnight cultures on BHI agar were washed with saline, centrifuged and resuspended in Tyrode's solution to a concentration of approximately 10^8 bacteria/ml.

Endotoxin

E. coli lipopolysaccharide (Difco) was dissolved in Tyrode's solution (20 μg/ml).

Pretreatment of mice

Bacteria, endotoxin or Tyrode's solution were injected intra-
peritoneally in a volume of 0.5 ml. Mice were sacrificed 30 minutes
later. Peritoneal cells were harvested as described above.

RESULTS

Observations on peritoneal cells in stained smears

At least 95% of the intact cells seen on stained smears prepared
from peritoneal washings of either normal or irradiated mice were mono-
nuclear and could be classified either as macrophages or as small or
medium sized lymphocytes. Mast cells, polymorphonuclear leucocytes and
occasional eosinophils accounted for about 5%, intact mesothelial cells
were found only rarely. Because of their small numbers, these cell types
were not included in the differential counts.

Disrupted cells, generally bare nuclei, were observed on all slides,
even in those areas of the slides where a minimum of distortion had
occurred. In preparations from normal animals about 13% of the cells
counted fell into the "broken" category. After irradiation, this per-
centage was increased. It is likely that many of the broken cells seen
on smears from irradiated mice were macrophages which had been disrupted
during preparation of the slides. This was suggested by the morphology
and staining characteristics of slightly damaged cells and by observa-
tions on smears from mice which had been injected with bacteria shortly
before sacrifice. In the latter, numerous bacteria were frequently

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seen either enmeshed in fragments of cytoplasm or free in the immediate area surrounding a nucleus.

Phagocytosis of injected bacteria was observed in cell preparations from both normal and irradiated mice. Intracellular bacteria were found in almost every cell identified as a macrophage. Occasionally only 2-3 bacteria could be seen inside a cell, more generally the cytoplasm seemed to be filled. Intracellular bacteria were also observed in the few polymorphonuclear leucocytes present, but infiltration of the peritoneal cavity with these cells had not yet occurred at the time of sacrifice (30 minutes after injection). Intracellular bacteria were not seen in cells classified as lymphocytes.

In addition, clumps of cells were always found in preparations from infected mice. Where sufficient detail could be discerned, the clumps consisted primarily of macrophages.

Observations on peritoneal cells under phase-contrast

Macrophages and lymphocytes were readily distinguished in peritoneal washings on the basis of morphology. For rapid differentiation with the phase-contrast microscope, the 40X objective proved to be more satisfactory than oil immersion.

Macrophages were round or slightly oval with an irregular outline. The nucleus appeared opaque; the cytoplasm contained many diffuse granules which were highly refractile. At a glance, the cell gave the impression of a very bright doughnut. Lymphocytes were uniformly spherical and had a well defined smooth outline. A narrow band of cytoplasm, distinctly

blue-green in appearance, surrounded the nucleus. Macrophages were 10-16 μ in diameter, lymphocytes measured 6-12 μ. Size, however, was not used as a distinguishing criterion. In cell suspensions obtained from normal mice and mice recovering from irradiation (30 or more days after exposure), the lymphocyte population consisted primarily of small cells (6-8 μ). In cell suspensions obtained during the first three weeks postirradiation, lymphocytes measuring 10-12 μ predominated.

Mast cells and polymorphonuclear leucocytes were always present in small numbers but were not included in the differential counts.

Comparison of differential counts by phase-contrast microscopy and in stained smears

The percentages of macrophages determined under phase-contrast and in stained smears are compared in Table I. In most instances, both differential counts were made on the same cell suspensions. In the case of normal mice and of mice sacrificed early (1-3 days) and late (30 days and thereafter) in the postirradiation period, excellent agreement was obtained by the two methods. However, between 5 and 21 days postirradiation, the percentage of macrophages was uniformly lower in stained smears than under phase. It is of interest that during this postirradiation period, an increase in the percentage of broken cells was found in all smears.

Phase differential counts, which could be made directly on the peritoneal washings with a minimum of handling, were probably more reliable, where discrepancies did exist, than counts made from smears.

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Consequently, the former were used to calculate the number of macrophages in all experiments.

Changes in total peritoneal cell counts and macrophage counts after irradiation

Table II and Figure I summarize data on peritoneal cells obtained from normal and X-irradiated mice which had not received any injections prior to sacrifice. No differences were observed in the response to the two X-ray doses employed. Therefore, 690 and 720 R experiments were combined.

It can be seen that mean total cell counts decreased sharply during the first 24 hours postirradiation and more gradually thereafter, reaching a minimum 21 days postirradiation. Leucocyte counts began to rise after the 3rd week and approached normal levels by the 12th week. The number of macrophages, as calculated from the percentage of these cells and the mean total cell counts, remained unchanged one day postirradiation. Thereafter a gradual decline occurred which reached a minimum 21 days postirradiation. Normal levels of macrophages were again attained 10-12 weeks after exposure to X-rays.

The viability of the cells recovered from the peritoneal cavity was essentially the same in all experiments. Average viability was 92%.

Effects of injection of bacteria or endotoxin on peritoneal cells of mice

Mean total leucocyte counts of mice which had been injected intraperitoneally with one of several agents 30 minutes before sacrifice are shown in Table III and Figure II. Data are presented for normal mice and for mice

7 days after exposure to either 690 or 720 R. Similar results were obtained at other times up to at least 30 days postirradiation.

In the unirradiated groups, mean total cell counts were not significantly different (P > 0.05) between uninjected mice and mice given either diluent (Tyrode's solution), suspensions of E. coli or S. aureus, or E. coli lipopolysaccharide. However, in irradiated mice, injection of any of these agents caused a highly significant decrease (P < 0.01) in the number of cells recovered from the peritoneal cavity. Among the four treated groups, the effects of diluent and S. aureus on the one hand, and E. coli and endotoxin on the other, were almost identical. Mean cell counts of E. coli and endotoxin treated mice were significantly lower (P < 0.05) than those of diluent-injected mice.

Attempts were made to determine the percentage of macrophages in the peritoneal cells of the injected mice. In diluent-treated mice, these percentages were almost identical to those found in uninjected mice (cf. Table I). However, in preparations from bacteria and endotoxin-treated mice, extensive clumping of cells (predominantly of macrophages) was observed and differential counts could only be estimated. These varied widely and were therefore not considered to be meaningful.

DISCUSSION

The experiments reported here involve only cell populations which were naturally present in the peritoneal cavity and not those which appeared as a result of prior peritoneal irritation. It is likely that cells found in an induced exudate differ in ways other than number and

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type from cells which occur normally. Indeed, it has been shown that certain properties of peritoneal cells varied with the agent used to elicit the exudate (10).

Our data on the number and types of peritoneal cells in normal LAF_1 mice are in general agreement with the findings of other investigators who studied different strains of mice (4, 5, 6, 7, 11).

After exposure to total-body X-irradiation, the most striking changes in the peritoneal cell population occurred within the first 24 hours. Total cell counts fell by more than 50% and proportions of macrophages and lymphocytes were greatly altered. The number of macrophages remained unchanged; the entire cell loss could be accounted for by the disappearance of lymphocytes. During the next 20 days postirradiation, the decrease in total cell count was very gradual and the ratio of the two types of mononuclear cells remained essentially constant (Tables I & II). It is of interest to note that macrophages far outnumbered all other cell types in the peritoneal cavity during this period.

During the 4th week postirradiation, percentages of cell types and total leucocyte counts began to return to normal. No explanation can be offered at present for the peak counts at 30 days. To date, the oldest survivors were tested 85 days postirradiation. At that time, the proportion of macrophages was the same as in normal mice. Total cell counts were only slightly lower than in unirradiated mice. Experiments are still in progress to determine if further recovery occurs after 85 days.

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The radiosensitivity of lymphocytes needs no further comment. The loss of large numbers of these cells from the peritoneal cavity immediately after irradiation was to be expected. On the other hand, peritoneal macrophages seemed relatively radioresistant and persisted as the dominant cell type after X-ray exposure. Their number was unaltered one day postirradiation and then decreased slowly to about 50% of normal during the next 20 days. Their viability, upon washing out of the cavity, was as high as that of cells from normal mice. Based on qualitative microscopic observations, they were able to ingest bacteria in vivo as well as did cells from unirradiated mice. However, the likelihood that the peritoneal macrophages had suffered some damage as a result of irradiation was suggested by their increased fragility.

Examination of stained smears prepared from peritoneal cells at various times postirradiation revealed that the percentage of broken cells was much higher from the 5th to the 21st day (Table I). Many of the broken cells had the appearance of macrophages which had been disrupted in preparation of the slides.

Peritoneal macrophages from irradiated mice may also be more sensitive to disturbances of their in vivo environment than cells from normal mice. During the postirradiation period, significantly fewer leucocytes (over 70% of which were macrophages) could be washed out of the peritoneal cavity of diluent or bacteria-injected mice than of uninjected mice. By contrast, in unirradiated mice, the cell yields for injected and uninjected groups did not differ statistically (Table III). Furthermore, after

injection of bacteria or endotoxin, far more severe clumping of cells (mostly of macrophages) was observed in washouts from irradiated than from unirradiated mice.

An adequate explanation cannot be offered at this time for the loss of cells from the peritoneal cavity of irradiated mice 30 minutes after injection of diluent. It is not likely that this was due to pyrogenicity of the solution. It is interesting to note that macrophages and lympho-cytes were affected equally since the proportions of these cell types remained unaltered. The greater reduction in cell counts after injec-tion of suspensions of E. coli or E. coli lipopolysaccharide may have been due to an increased sensitivity of the peritoneal fluid cells to the effects of bacterial endotoxin.

Another factor which may have reduced the number of cells which could be washed out of the peritoneal cavity of E. coli or endotoxin-treated mice was extensive clumping of the macrophages. However, a similar degree of clumping was observed in peritoneal washings from mice injected with S. aureus, yet cell counts of these mice did not differ from those of diluent-injected mice where clumping was not a complicat-ing factor. Other investigators (12, 13, 14) also reported clumping and decreases in mononuclear peritoneal cells following administration of particulate matter or endotoxin, but they made their earliest observa-tions 1 or more hours after injection and did not compare the response in normal and irradiated animals.

The experiments reported here indicate that peritoneal macrophages survived total body X-irradiation and were able to function phagocytically. However, our observations also suggest that these cells suffered some injury which resulted in greater fragility as soon as their natural environment was disturbed. Similar conclusions were reached by Balner (7) who found that macrophages from irradiated mice were more susceptible to the cytotoxic action of indifferent serum than were cells from normal mice. The nature of this latent radiation damage warrants further study, especially with respect to intracellular digestion of phagocytized microorganisms.

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TABLE I

PERCENTAGES OF MACROPHAGES IN PERITONEAL CELLS OF NORMAL AND X-IRRADIATED MICE AS DETERMINED UNDER PHASE CONTRAST AND IN STAINED SMEARS

	NO. OF EXPERIMENTS*	PHASE CONTRAST % MACROPHAGES	STAINED SMEARS % MACROPHAGES	% "BROKEN"
NORMAL	7 (8)	29.1	29.6	12.8
DAY POSTIRRADIATION**				
1	6 (6)	65.3	62.0	17.0
3	4 (6)	65.8	61.1	16.6
5	11 (3)	70.8	53.4	21.6
7	5 (5)	73.8	58.8	24.7
10	5 (3)	72.0	53.8	29.2
12	3 (3)	73.0	72.5	13.6
14	2 (2)	73.5	63.0	31.8
21	2 (2)	73.5	58.8	28.7
30	5 (4)	66.6	67.9	9.5
42	3 (2)	63.3	66.5	6.5
53	2 (1)	56.5	47.5	12.5
73	2 (1)	43.0	41.0	11.0
85	3 (2)	30.7	30.0	9.0

*First figure refers to phase counts; figure in parenthesis indicates experiments for which stained smears were examined.
**690 and 720 R groups combined.

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TABLE II

TOTAL PERITONEAL LEUCOCYTE AND MACROPHAGE COUNTS OF NORMAL AND X-IRRADIATED MICE

	NUMBER OF EXPERIMENTS	LEUCOCYTES/MOUSE*	MACROPHAGES/MOUSE**
		$x\ 10^6$	$x\ 10^6$
NORMAL	8	7.4 ± 0.7	2.2
DAY POSTIRRADIATION***			
1	6	3.4 ± 0.2	2.2
3	6	2.6 ± 0.1	1.7
5	5	2.6 ± 0.2	1.8
7	5	2.3 ± 0.03	1.7
10	3	2.1 ± 0.4	1.5
12	3	1.7 ± 0.2	1.3
14	2	1.7 ± 0.1	1.2
21	2	1.5 ± 0.3	1.1
30	5	3.1 ± 0.2	2.1
42	3	2.6 ± 0.1	1.7
53	2	3.5 ± 0.3	2.0
73	2	5.3 ± 0.7	2.3
85	3	6.7 ± 0.5	2.1

*Mean ± Standard Error.
**Mean leucocyte count x percentage macrophages (phase).
***690 and 720 R groups combined.

TABLE III

PERITONEAL CELL COUNTS OF NORMAL AND X-IRRADIATED MICE BEFORE AND 30 MINUTES AFTER INJECTION OF DILUENT, BACTERIA OR ENDOTOXIN

	NORMAL MICE		7 DAYS POSTIRRADIATION**	
	NO. OF EXPERIMENTS	LEUCOCYTES/ MOUSE*	NO. OF EXPERIMENTS	LEUCOCYTES/ MOUSE*
		$x\ 10^6$		$x\ 10^6$
UNINJECTED	8	7.4 ± 0.8	5	2.3 ± 0.03
DILUENT	4	7.1 ± 1.1	4	$1.4 \pm 0.2^{\Delta}$
S. aureus	4	6.9 ± 0.7	4	$1.3 \pm 0.2^{\Delta}$
E. coli	6	6.3 ± 1.5	5	$0.8 \pm 0.1^{\Delta+}$
E. coli ENDOTOXIN	4	6.0 ± 0.3	4	$0.9 \pm 0.1^{\Delta+}$

*Mean ± Standard Error.
**690 and 720 R groups combined.
Δt test for difference between means significant at $P < 0.01$ (difference between irradiated uninjected and experimental group).
+t test for difference between means significant at $P < 0.05$ (difference between irradiated diluent-injected and experimental group).
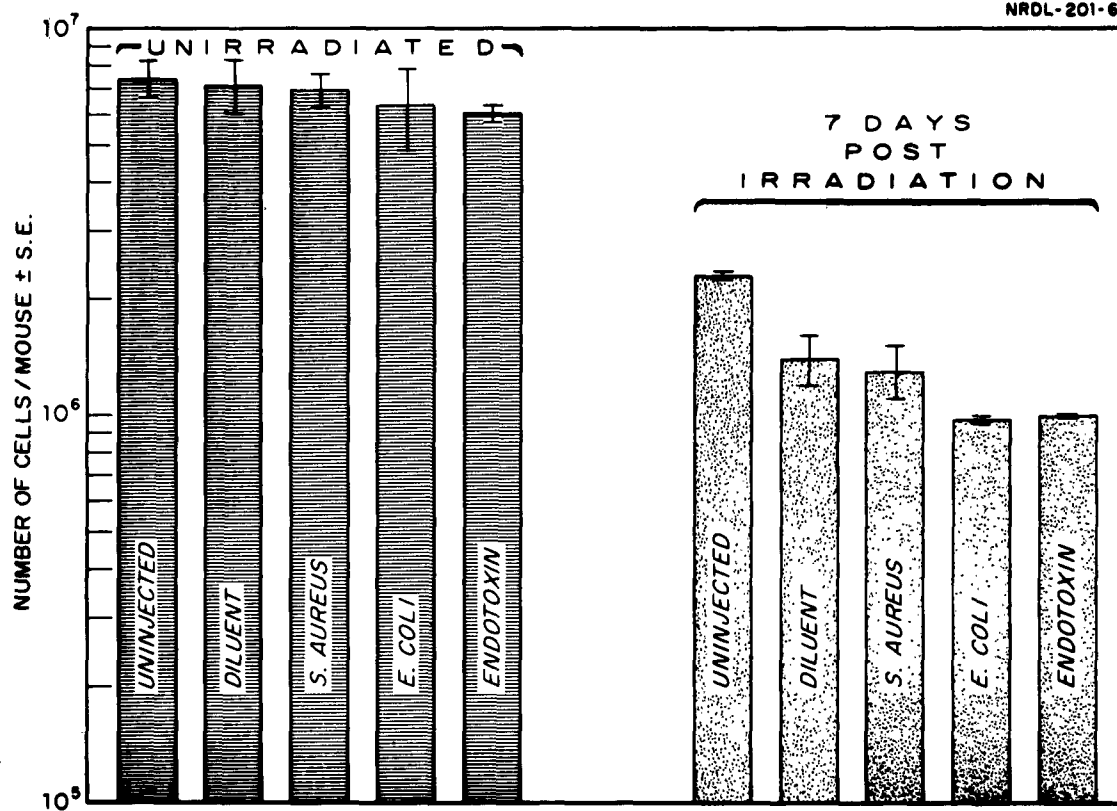
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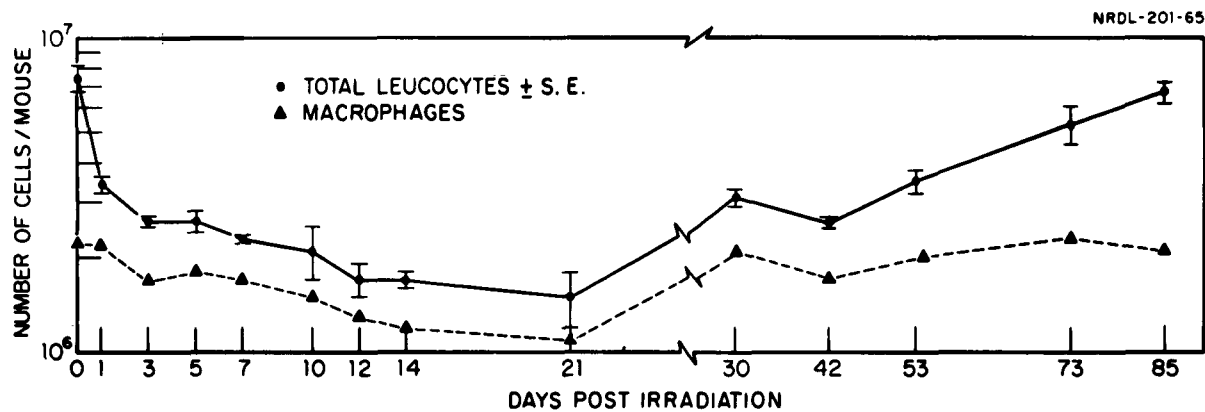Figure I. Total peritoneal leucocyte and macrophage counts of normal and X-irradiated mice.

Figure II. Peritoneal cell counts of normal and X-irradiated mice before and 30 minutes after injection of diluent, bacteria or endotoxin.

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	Bureau of Medicine and Surgery Washington, D. C. 20390, and Defense Atomic Support Agency, Washington, D. C. 20301

13. ABSTRACT Peritoneal cells of normal and irradiated LAF_1 mice which had not been pretreated with peritoneal irritants were studied by phase-contrast microscopy and in stained smears. The average number of leucocytes recovered per normal mouse was 7.4×10^6. Approximately 95% of the cells were mononuclear; of these, macrophages comprised 29% (2.2×10^6), small and medium lymphocytes 71% (as determined by phase-contrast). One day after whole-body exposure to 690 R ($LD_{30/30}$) or 720 R ($LD_{50/30}$), the mean leucocyte count was 3.4×10^6, macrophages accounted for 65% (2.2×10^6) of the cells. From 3 to 21 days postirradiation, cell counts gradually decreased further to 1.5×10^6. Macrophages decreased at the same rate; they constituted approximately 79% of the cells during this period. Thereafter, the number of leucocytes increased and reached normal levels by the 12th week postirradiation.

When suspensions of viable Escherichia coli or Staphylococcus aureus were injected 30 minutes before sacrifice, the macrophages of both normal and irradiated mice contained large numbers of microorganisms. Thirty minutes after injection of bacteria or E. coli lipopolysaccharide (10 µg) into normal mice, the number of cells in peritoneal washings was not altered. However, after injection of these agents into irradiated mice, total cell counts were significantly lower than in uninjected or diluent-injected mice.

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14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Peritoneal cells						
X-irradiation						
Macrophages						

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